Effects of N-Carbamylglutamate and Arginine on Steroidogenesis and Proliferation of Porcine Granulosa Cells in Vitro

A.A. DeVore, T. Feng, L. J. Spicer

Honors Thesis

Department of Animal Science, Oklahoma State University,

Stillwater, OK 74078

Abstract

In vivo studies have determined that dietary N-carbamylglutamate (NCG) and arginine (ARG) can enhance reproductive performance in gilts, ewes and rats. We hypothesized that both NCG and ARG will enhance hormone-induced estradiol (E2) production by granulosa cells, explaining why these compounds could improve reproductive performance in pigs. Therefore, the objective of these studies was to evaluate the direct effects of NCG and ARG on porcine granulosa cell proliferation and steroidogenesis, using an *in vitro* cell culture system. Granulosa cells from small (1-5 mm) and large (>5 mm) porcine follicles were cultured for 2 days in 5% fetal bovine serum and 5% porcine serum-containing medium followed by 2 days in serum-free medium containing 500 ng/ml of testosterone, and NCG or ARG at various doses in the presence of either follicle-stimulating hormone (FSH; 30 ng/ml), insulin-like growth factor-1 (IGF1; 30 ng/ml), or both. Numbers of granulosa cells were determined at the end of the experiment using a Coulter counter and concentrations of progesterone (P4) and E2 in culture medium were determined using radioimmunoassays. Our results indicated that large-follicle granulosa cells (LGGC) were more responsive to NCG and ARG than small-follicle granulosa cells (SMGC). Specifically, NCG and ARG significantly decreased IGF1 plus FSH induced P4 production, whereas E2 production and cell proliferation were not significantly affected in SMGC. In LGGC, NCG inhibited ($P < 0.05$) basal and FSH-induced P4 and E2 production but stimulated cell numbers; whereas ARG inhibited FSH-induced E2 production and cell numbers. These studies indicate that NCG and ARG directly affect follicular function in the pig.

Key Words: N-Carbamylglutamate (NCG), Arginine (ARG), Granulosa cell, Proliferation, Steroidogenesis, Insulin-like growth factor-1 (IGF1), Follicle-stimulating hormone(FSH)

Introduction

In the swine industry, reproductive performance plays a pivotal role in gilt and sow productivity. The number of piglets produced per sow has increased as technological advances have been implemented into raising animal for food production. This has been accomplished through artificial insemination, controlled environment and nutrient supplementation.

N-carbamylglutamate (NCG), is a structural analogue of N-acetylglutamate, the primary enzyme of the L-arginine (ARG)-synthetic pathway (Wu et al., 2004), allowing pigs to synthesize ARG more efficiently. ARG is an essential amino acid particularly important for the growth of piglets (Wu and Morris, 1998). Dietary supplementation of ARG has a multifaceted role in treating a multitude of health and developmental problems in pigs (Wu et al., 2009). Recent studies have shown NCG-supplementation throughout the first 60 days of gestation improves pregnancy outcome in gilts and is associated with improved concentrations of NCG in plasma and placental angiogenic factors, whereas in late gestating sows, ARG supplementation had no effect on litter size, number born alive, or birth weight (Zhang et al., 2014). Previous studies have shown that NCG supplementation in late gestation sows increased litter size, number born alive, and birth weights of piglets (Wu et al., 2012a). Similrly, in Awassi sheep (Al-Dubbas et al. 2008) and rats (Pau and Milner, 1982), ARG supplementation increased ovulation rate. ARG supplementation increased fertility but did not alter ovulation rate in Suffolk ewes (de Chevez et al. 2015). While in high-yielding dairy cattle, NCG supplementation increases the metabolizable protein utilization, thereby increasing lactation

performance (Chacher et al., 2014) which could indirectly impact reproductive performance. In mares, ARG supplementation increased follicle diameter but did not influence interovulatory interval (Kelly et al., 2014). In women, ARG supplementation decreased the number of follicles and pregnancy rates after in-vitro fertilization (IVF) (Battaglia et al., 2002), but whether ARG directly or indirectly affects follicular function is uncertain. Currently no studies have been conducted to investigate the effects of ARG and NCG on porcine granulosa cell function. Therefore, our objectives were to determine if ARG and NCG can directly affect steroidogenesis and proliferation of porcine granulosa cells.

Materials and Methods

Reagents and Hormones

The reagents used in cell culture were: DMEM, hyaluronidase, pronase, DNase, collagenase, sodium bicarbonate, ARG, NCG, and trypan blue obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO); pig serum (PS) and fetal calf serum (FCS) were obtained from Atlanta Biologicals (Flowery Branch, GA). Purified ovine follicle-stimulating hormone (FSH) (FSH activity: 15 x NIH-FSH-S1 U/mg) from the National Hormone and Pituitary Program (Torrance, CA), testosterone from Steraloids (Wilton, NH) and recombinant human insulin-like growth factor-1 (IGF1) from R&D Systems (Minneapolis, MN).

Cell Culture

Ovaries were collected from non-pregnant gilts slaughtered at Oklahoma State University Food and Agricultural Products Center and based on surface diameter, granulosa cells were collected from small (1 to 5 mm) or large (> 5 mm) follicles as described previously (Ranzinego et al., 2008). Granulosa cells were resuspended with a basal DMEM medium (containing 0.12

mM of gentamicin and 38.5 mM of sodium bicarbonate) containing collagenase at 1.25 mg/mL and DNase at 0.5 mg/mL to prevent cell clumping. Viability of granulosa cells was determined via Trypan blue exclusion method, and averaged 36.5%, which is within the range previously reported for porcine granulosa cells (Evans et al., 2014).

Approximately 2.6 $x10^5$ viable cells were plated on 24-well Falcon mutilwell plates (Becton Dickinson, Lincoln Park, NJ, USA). Cells were cultured in an environment of 5% CO_2 and 95% air at 38.5 °C in 5% FCS and 5% PS for the first 48 h with medium changed every 24 h. Cells were then washed twice with 0.5 ml of serum-free medium and the various treatments applied in serum-free medium for 48 h. Then medium was collected for steroid radioimmunoassays (RIA) and cells were enumerated.

Steroid Radioimmunoassays (RIA) and Cell Counting

Progesterone (P4) and estradiol (E2) RIA were conducted as previously described (Spicer and Chamberlain, 1998; Evans et al., 2014). Intra-assay coefficients of variation averaged 7.1% for the P4 and 8.6% for the E2 RIA.

To determine cell numbers, cells were washed and treated with trypsin (2.5%) for 20 min at 37°C. Cells were transferred into 10 ml of saline before being counted using a Coulter counter (Z2 Coulter® Particle Count and Size Analyzer; Beckman Coulter, Hialeah, FL) as previously described (Langhout et al., 1991; Lagaly et al., 2008; Ranzinego et al., 2008).

Experimental Design

Experiment 1 was designed to evaluate the effects of NCG and ARG on the proliferation and steroidogenesis of porcine granulosa cells from small-follicles (SMGC). Cells were cultured as stated before and the following treatments were applied for 48 h: NCG (0, 0.4 or 4 mM) and

ARG (0, 0.4, or 4 mM) in the presence of IGF1 (30 ng/mL) and testosterone (500 ng/mL) with or without FSH (30 ng/mL). The doses of IGF1 and FSH were based on previous studies (Ranzinego et al., 2008; Spicer et al., 2002). The doses of NCG and ARG were selected based on previous studies (Lamanna et al., 2007; Greene et al., 2013). Cells were counted and RIA was conducted to measure P4 and E2 concentrations in medium.

Experiment 2 was designed to evaluate the effect of NCG and ARG on the proliferation and steroidogenesis of porcine granulosa cells from large-follicles (LGGC). Cells were cultured as stated before and the following treatments were applied for 48 h: NCG (0 or 4 mM) and ARG (0 or 4 mM) in the presence of FSH (30 ng/mL), IGF1 (30 ng/mL) and testosterone (500 ng/mL). Dosage of NCG and ARG were selected based on the results from Experiment 1.

Statistical Analysis

For each experiment three, different pools of granulosa cells were used as experimental (i.e., biological) replicates, and each treatment was replicated 3 or 4 times in each experiment. Granulosa cells in each pool was generated from a total volume of 1 to 6.5 ml of follicular fluid (from 2 to 10 ovaries) per pool. Cell numbers from each experiment were calculated to determine steroid production as ng or pg /10^5 cells per 24 h. Treatment effects on the dependent variables were determined using ANOVA and the general linear models (GLM) procedure of SAS for Windows (Version 9.3, SAS Institute Inc., Cary, NY). Data from Experiment 1 were analyzed as a one-way ANOVA. Data from Experiment 2 were analyzed as a 2 x 3 factorial ANOVA. Mean differences were determined using the Fisher's protected least significant differences test (Ott, 1977) only if significant main effects in the ANOVA were detected. Data are presented as least square means ± SEM.

Results

Experiment 1: Effect of NCG and ARG on SMGC steroidogenesis and cell proliferation

In SMGC, 4 mM of NCG and ARG decreased ($P < 0.05$) IGF1 plus FSH-induced P4 production by 31.5% and 19.8%, respectively (Fig. 1A). In IGF1-treated SMGC, ARG and NCG had no effect ($P > 0.10$) on P4 production whereas FSH increased ($P < 0.05$) IGF1-induced P4 production by 3.1-fold (Fig. 1A).

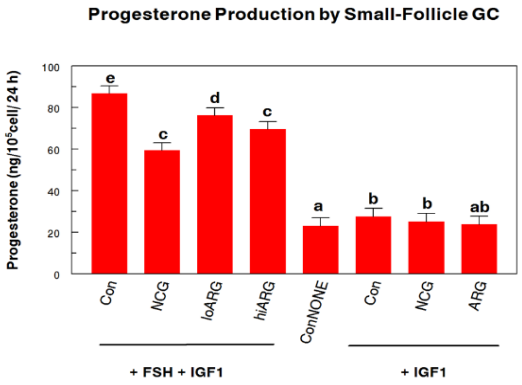
In SMGC, NCG and ARG did not affect ($P > 0.10$) FSH plus IGF1-induced E2 production or IGF1-induced E2 production (Fig. 1B). IGF1-induced E2 production was increased ($P < 0.05$) by 2.4-fold in the presence of FSH (Fig. 1B).

Numbers of SMGC were not significantly affected by NCG, ARG or IGF1. FSH increased ($P < 0.05$) cell numbers by 1.3-fold in IGF1-treated SMGC (Fig. 1C).

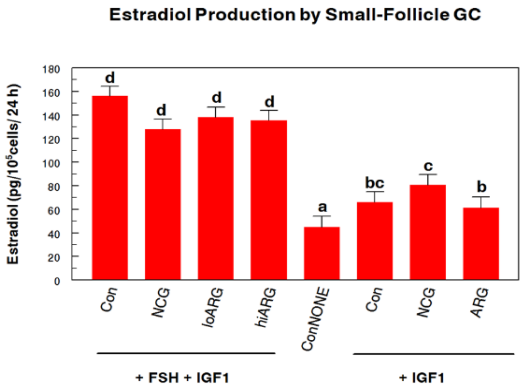
Figure 1: The effects of ARG and NCG on proliferation and E2 and P4 production by SMGC. Over a 48 h period, cells were treated with NCG or ARG and concomitantly treated with testosterone (500 ng/mL) and IGF1 (30 ng/mL), with or without FSH (30 ng/mL). **abcde** Within a panel, means without a common letter differ ($P < 0.05$).

Figure 1

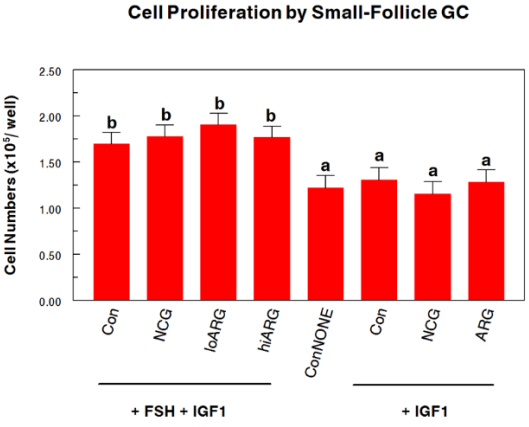
A


Progesterone Production by Small-Follicle GC
Progesterone (ng/10^5cell/ 24 h)
100
80
60
40
20
0
e
c
d
c
a
b
b
ab
Con
NCG
loARG
hiARG
ConNONE
Con
NCG
ARG
+ FSH + IGF1
+ IGF1

B


Estradiol Production by Small-Follicle GC
Estradiol (pg/10^5cells/ 24 h)
180
160
140
120
100
80
60
40
20
0
d
d
d
d
a
bc
c
b
Con
NCG
loARG
hiARG
ConNONE
Con
NCG
ARG
+ FSH + IGF1
+ IGF1

C


Cell Proliferation by Small-Follicle GC
Cell Numbers (x10^5/ well)
2.50
2.00
1.50
1.00
0.50
0.00
b
b
b
b
a
a
a
a
Con
NCG
loARG
hiARG
ConNONE
Con
NCG
ARG
+ FSH + IGF1
+ IGF1

Experiment 2: Effects of NCG and ARG on LGGC steroidogenesis and cell proliferation

In LGGC treated with IGF1 alone, NCG decreased ($P < 0.05$) P4 production by 36%, whereas FSH increased P4 production by 18% and ARG had no effect ($P > 0.10$) on P4 production (Fig. 2A). Similarly, in LGGC treated with FSH and IGF1, NCG decreased ($P < 0.05$) P4 production by 46% whereas ARG had no effect ($P > 0.10$) on P4 production (Fig. 2A).

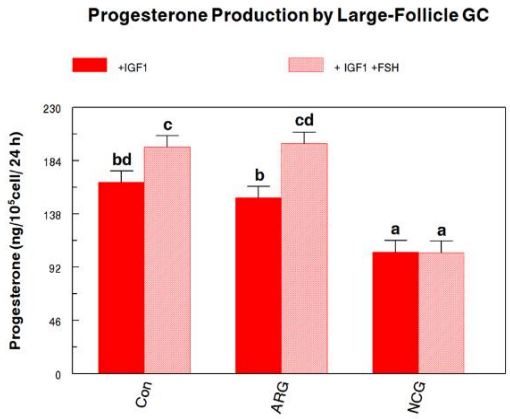
In LGGC treated with IGF1 alone, NCG decreased ($P < 0.05$) E2 production by 46% whereas ARG had no significant effect on E2 production and FSH increased ($P < 0.05$) E2 production by 1.7-fold (Fig. 2A). In LGGC treated with FSH and IGF1, ARG and NCG decreased ($P < 0.05$) E2 production 26% and 63%, respectively (Fig. 2B).

In LGGC treated with IGF1 alone, NCG increased ($P < 0.05$) cell numbers by 1.4-fold and FSH increased ($P < 0.05$) cell numbers by 1.5-fold, whereas ARG had no significant effect (Fig. 2C). In LGGC treated with FSH plus IGF1, ARG decreased ($P < 0.05$) cell numbers by 20% whereas NCG was without effect ($P > 0.10$) (Fig. 2C).

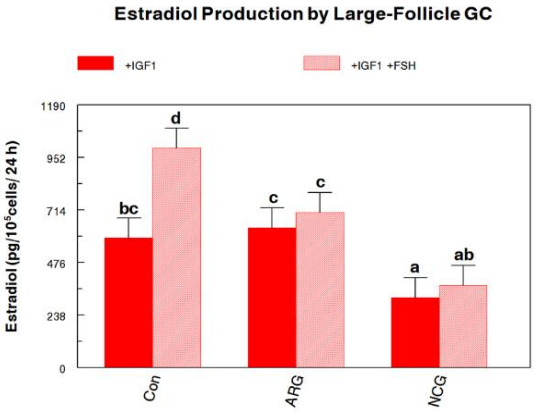
Figure 2: The effect of NCG and ARG on proliferation and E2 and P4 production by LGGC. Over a 48 h period, cells were treated with NCG or ARG and concomitantly treated with testosterone (500 ng/mL) and IGF1 (30 ng/mL), with or without FSH (30 ng/mL). **[abcd]** Within a panel, means without a common letter differ ($P < 0.05$).

Figure 2

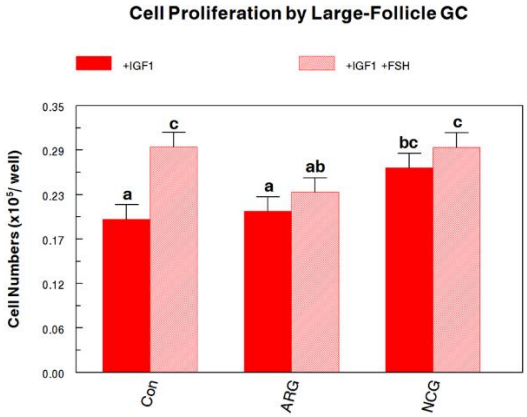
A


Progesterone Production by Large-Follicle GC
+IGF1
+ IGF1 +FSH
Progesterone (ng/10^5cell/ 24 h)
230
184
138
92
46
0
bd
c
b
cd
a
a
Con
ARG
NCG

B


Estradiol Production by Large-Follicle GC
+IGF1
+IGF1 +FSH
Estradiol (pg/10^5cells/ 24 h)
1190
952
714
476
238
0
bc
d
c
c
a
ab
Con
ARG
NCG

C


Cell Proliferation by Large-Follicle GC
+IGF1
+IGF1 +FSH
Cell Numbers (x10^5/ well)
0.35
0.29
0.23
0.17
0.12
0.06
0.00
a
c
a
ab
bc
c
Con
ARG
NCG

Discussion

This study demonstrates for the first time the effects of ARG and NCG on steroidogenesis and cell proliferation in ovarian granulosa cells of a multi-ovular species. Formerly, the effects of ARG and NCG on ovarian cell steroidogenesis and proliferation had been minimally studied. Previously, a combined treatment of FSH and IGF1 increases production of E2 and P4 in cultured porcine granulosa cells (Ranzinego at al., 2008), and the present study confirmed this. Our results also show that IGF1 increased E2 or P4 production slightly in the absence of FSH. Estradiol and P4 concentrations increase as granulosa cells undergo differentiation (Hsueh et al., 1983); therefore, E2 and P4 production was assessed in this study. Previously, pregnant ewes that were given ARG and NCG as a dietary supplement exhibited a significant reduction in blood levels of P4 and E2 (Zhang et al., 2016). In our study, only FSH plus IGF1-induced P4 production was significantly reduced when treated with ARG and NCG in SMGC without affecting E2 production. In contrast, P4 and E2 production were inhibited by both ARG and NCG in LGGC, suggesting that ARG and NCG effects are more pronounced in more differentiated LGGC. In porcine testicular tissue ARG (1 mM) caused a decrease in basal testosterone production (Lamanna et al., 2007). Why NCG and ARG may suppress steroidogenesis of highly differentiated cells more than less differentiated cells will require further study.

For the first time, cell proliferation of porcine granulosa cells treated with NCG and ARG has been studied. Specifically, the present study found no significant effect of NCG and ARG on proliferation of porcine SMGC, whereas NCG increased numbers of LGGC in the absence of FSH. In the presence of IGF1, FSH increased numbers of both SMGC and LGGC. In agreement with the present study, Khamsi and Armstrong (1997) showed that FSH increases bovine

granulosa cell proliferation in the presence of IGF1. However, FSH had no effect on basal or insulin-induced proliferation of porcine granulosa cells (Hammond and English,1987). Previously, NCG increased measures of intestinal epithelium cell proliferation in weanling piglets (Wu et al., 2012b). A previous study reported the role of NCG and ARG on some microRNA gene expression and their effects on the size and volume flow of the porcine umbilical vein (Liu et al., 2011), supporting a biological effect for these two amino acid derivatives. Our results indicate that NCG and ARG affect granulosa cell differentiation more than granulosa cell proliferation. Why ARG and NCG had opposing effects on cell proliferation of highly differentiated granulosa cells will require further study.

Our results imply that ARG and NCG could be used as nutritional supplements to help regulate ovarian function in pigs by inhibiting P4 production in SMGC and E2 production in LGGC. Understanding the mechanism of action of NCG and ARG through further research will allow us to understand how these nutritional supplements can improve reproduction in pigs and other species.

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